

Localization and content of nerve growth factor in peripheral blood eosinophils of atopic dermatitis patients

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Summary

Background Atopic dermatitis (AD) is a chronic and relapsing eczematous skin disorder characterized by eosinophilia. Nerve growth factor (NGF) modulates the allergic response through interactions with immune-inflammatory cells. Eosinophils have been reported to store NGF as a preformed mediator.

Objective To gain further insight into the significance of eosinophils in association with NGF in the pathogenesis of AD, the localization of NGF within eosinophils and the difference of the eosinophil-derived NGF content in the peripheral blood of normal volunteers vs. AD patients were investigated.

Methods We examined the localization of NGF within human eosinophils using the post-embedding immunoelectron microscopy and compared NGF content in freshly isolated eosinophil sonicates from the peripheral blood of 31 normal volunteers vs. 42 AD patients by immunoenzymatic assay. A possible correlation between the levels of NGF and major basic protein was also examined.

Results Immunoelectron microscopic studies revealed that NGF was localized in the central core of normal eosinophil granules, where major basic protein is also present as a preformed mediator, in homogeneous granules and in intergranular ductal or vesicular structures adjacent to specific granules of eosinophils. NGF content in eosinophils was significantly increased in AD patients. Furthermore, there was a significant correlation between levels of NGF and major basic protein in eosinophils of AD patients.

Conclusions Increased levels of NGF contained in eosinophils of the peripheral blood from AD patients, when released with other mediators such as basic proteins, could promote inflammation and local tissue damage.

Keywords atopic dermatitis, eosinophils, immunoelectron microscopy, major basic protein, nerve growth factor

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Introduction

Nerve growth factor (NGF) is the best-characterized member of the neurotrophin family; it plays a crucial role in the survival, development, differentiation and normal function of neurons [1]. There is increasing evidence that NGF, in addition to its actions within the nervous system, elicits a number of biologic effects on non-neuronal cells of the immune-inflammatory compartment, indicating that NGF might also play a role in allergic, inflammatory and autoimmune diseases. In fact, NGF causes hypertrophy of mast cells in newborn mice [2] and the massive degranulation of rat peritoneal mast cells [3]. Moreover, NGF enhances the survival, phagocytosis and superoxide production of mature murine neutrophils, causes mediator release from human

basophils, and induces the proliferation and differentiation of human B lymphocytes [4–6].

Atopic dermatitis (AD) is a chronic and relapsing eczematous skin disorder mostly associated with elevated IgE levels, eosinophilia and mast cell hyperplasia. A predominance of Th-2 cells has been found in the lesional skin and peripheral blood of AD patients, which may, at least in part, explain the inflammation in that disease [7]. Therefore, eosinophils, as well as mast cells and Th-2 cells, play central roles in the inflammation of AD. Mast cells and Th-2 cells are able to synthesize, store and release NGF [8, 9]. Solomon et al. [10] demonstrated that human eosinophils also store NGF as a preformed mediator that can activate eosinophils to release inflammatory mediators selectively in an autocrine manner. In addition, we recently reported that AD patients have significant increases in the plasma levels of NGF, and that the NGF plasma levels in AD patients significantly correlate with the disease severity of AD [11], which indicates a role of NGF in the pathogenesis of AD.

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Eosinophils are the predominant inflammatory cells of the late phase and chronic development of allergic inflammation. Activated eosinophils secrete many inflammatory and pro-inflammatory mediators that contribute to tissue injury and inflammation through a number of mechanisms, including the direct toxic action of basic proteins (major basic protein (MBP), eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase), the production of sulphido-peptide leukotrienes, the generation of toxic oxygen products and the release of cytokines [12].

To gain further insight into the significance of eosinophils in association with NGF in the pathogenesis of AD, in this study, we examined the localization of NGF within eosinophils and compared the eosinophil-derived NGF content in the peripheral blood of normal volunteers vs. AD patients, along with its possible correlation with MBP content.

Subjects and methods

Subjects

A total of 42 patients (26 males, 16 females; mean age, 24.9 years) with a confirmed diagnosis of AD according to the criteria of Hanifin and Rajka [13] were enrolled in this study. All patients had blood eosinophilia (range, 15–40%). The AD patients had not received any topical treatment for at least 2 weeks prior to the study or any systemic treatment for 1 month prior to the study. In addition, 31 healthy volunteers (18 males, 13 females; mean age 27.0 years) with no history of atopic disease served as the control group. None of the control subjects had any other concomitant dermatological or medical disorders. This study was conducted according to the ethical standards of the Toyama Medical and Pharmaceutical University (Japan), which require informed consent from each subject.

Isolation of eosinophils

We obtained venous blood (100–150 mL) in heparinized syringes from the antecubital vein of each subject, and eosinophils were purified by density fractionation centrifugation. Venous blood was mixed 5 : 1 with 4.5% dextran (Sigma Chemicals, St Louis, MO, USA) in 0.9% NaCl and was sedimented for 45 min at 22 °C. The white blood cell layer was collected, divided into 30 mL aliquots, and layered over 10 mL Ficoll–Hypaque (density 1.077 g/mL) (Sigma Chemicals) in 50-mL tubes. Cells were then centrifuged at 400 *g* for 20 min at 22 °C. The pellets containing leucocytes were resuspended in Ca²⁺-free Hank's balanced salt solution (HBSS), washed twice and then centrifuged at 400 *g* for 10 min. Cells were then suspended in HBSS containing 1 mmol/L Ca²⁺ and 5% FCS and diluted to a concentration of 2 × 10⁷/mL. In separate 15-mL tubes, discontinuous Percoll gradients were prepared (from top to bottom in g/mL [mL]: 1.080 [2.5]; 1.085 [2.5]; 1.090 [3.0]; 1.095 [3.0]; 1.100 [1.5]) in HBSS, over which 2 mL of the cell suspension was layered. Gradients were then centrifuged at 700 *g* for 20 min. The interface containing eosinophils (1.095–1.100 g/mL) was collected, diluted in HBSS, then centrifuged at 400 *g* for 10 min and resuspended in Dulbecco's modified Eagle's

medium (DMEM). Eosinophils were counted and assessed for purity by Wright–Giemsa stain. In this method, eosinophils were collected at a purity of 94–97%.

Immunocytochemical and immunoelectron microscopic staining

Immunocytochemistry was performed using an enhanced labelled polymer system conjugated to anti-mouse and anti-rabbit immunoglobulins (Dako, Kyoto, Japan), following the protocol of the manufacturer. Blood smears of each subject on glass slides were incubated overnight with a mouse anti-human NGF monoclonal antibody (Boehringer Mannheim, Mannheim, Germany), which belongs to the mouse immunoglobulin subclass immunoglobulin G1 (IgG1), at the pre-determined optimal dilution at 4 °C in a humidified atmosphere. After three 5-min washes in PBS, the slides were incubated with labelled-polymer for 30 min. The specimens were then reacted with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Dojin, Kumamoto, Japan) diluted in 0.05 M Tris buffer (pH 7.6) containing 10 mM hydrogen peroxide for 10 min at room temperature. Methyl green (Dako) was used as the counterstain.

The pellets containing eosinophils were prepared for immunoelectron microscopy by fixation in paraformaldehyde and were embedded in LR-White resin. Sections were incubated with the NGF antibody and were then subjected to reaction with 10 nm colloidal gold-conjugated goat anti-rabbit IgG (Zymed Laboratory, Inc., Co., San Francisco, CA, USA). Controls examined consisted of smears or sections in which the primary antibody was omitted and/or was substituted by MOPC (IgG1; Sigma, Deisenhofen, Germany) at the same concentration as the isotype control monoclonal antibody. The specificity of the reaction was also checked by incubating smears or sections with the primary antibody added to a 1000-fold excess of the antigen.

Determination of NGF and MBP contents in eosinophils

To determine the NGF content of eosinophils, freshly isolated eosinophil sonicates (1 × 10⁶/mL in DMEM) were prepared by continuous sonication for 1 min on ice (Heat Systems Ultrasonics, 50% duty cycle, output 5). NGF levels were measured in the eosinophil sonicates using a highly sensitive, two-site immunoenzymatic assay (ELISA) that recognizes human and murine NGF and is capable of detecting concentrations of NGF as low as 5 pg/mL. All procedures were performed using a commercial ELISA kit for NGF, following a modification of the protocol described by Boehringer Mannheim (Germany). Briefly, polystyrene 96-well microtube immunoplates were coated with affinity-purified monoclonal goat anti-NGF antibody for 2 h at room temperature. Parallel wells were coated with pre-immune goat IgG for evaluation of non-specific signals. Samples and standard solutions (0–1000 pg/mL) were added and the plates were incubated overnight at 4 °C. After washing, an NGF monoclonal antibody conjugated to 400 mU/mL β-galactosidase (Boehringer Mannheim) was added and the microtitre plates were incubated overnight at 4 °C. The enzyme substrate chlorophenol-red-α-D-galactopyranoside (2 mg/mL) (Boehringer Mannheim) was then added and the plates were

incubated for 8 h at room temperature. The optical density of each well was measured at 575 nm using an ELISA reader and the values of standards and samples were corrected by subtracting the background value due to non-specific binding. Data are reported as pg/mL; all assays were performed in triplicate for each subject. MBP levels in eosinophil sonicates were evaluated using a radioimmunoassay (RIA), as previously described in detail [14].

Statistical analysis

Results are reported as mean values $\pm$ SD. The unpaired Student's *t*-test was used to determine the statistical significance of differences between groups of AD patients and control subjects. The Spearman rho test was performed to determine whether a significant correlation exists between

NGF and MBP contents in isolated blood eosinophils as well as between the NGF content and the number of eosinophils. A probability of 0.05 or less was considered statistically significant.

Results

Localization of NGF in eosinophils

All eosinophils, as identified by Wright–Giemsa staining, were clearly labelled by affinity-purified NGF antibodies in smears of the peripheral blood. The highest levels of immunoreactivity appeared to be localized within eosinophil granules (Fig. 1a). A quite small number of mononuclear lymphoid cells that were sometimes intermingled showed

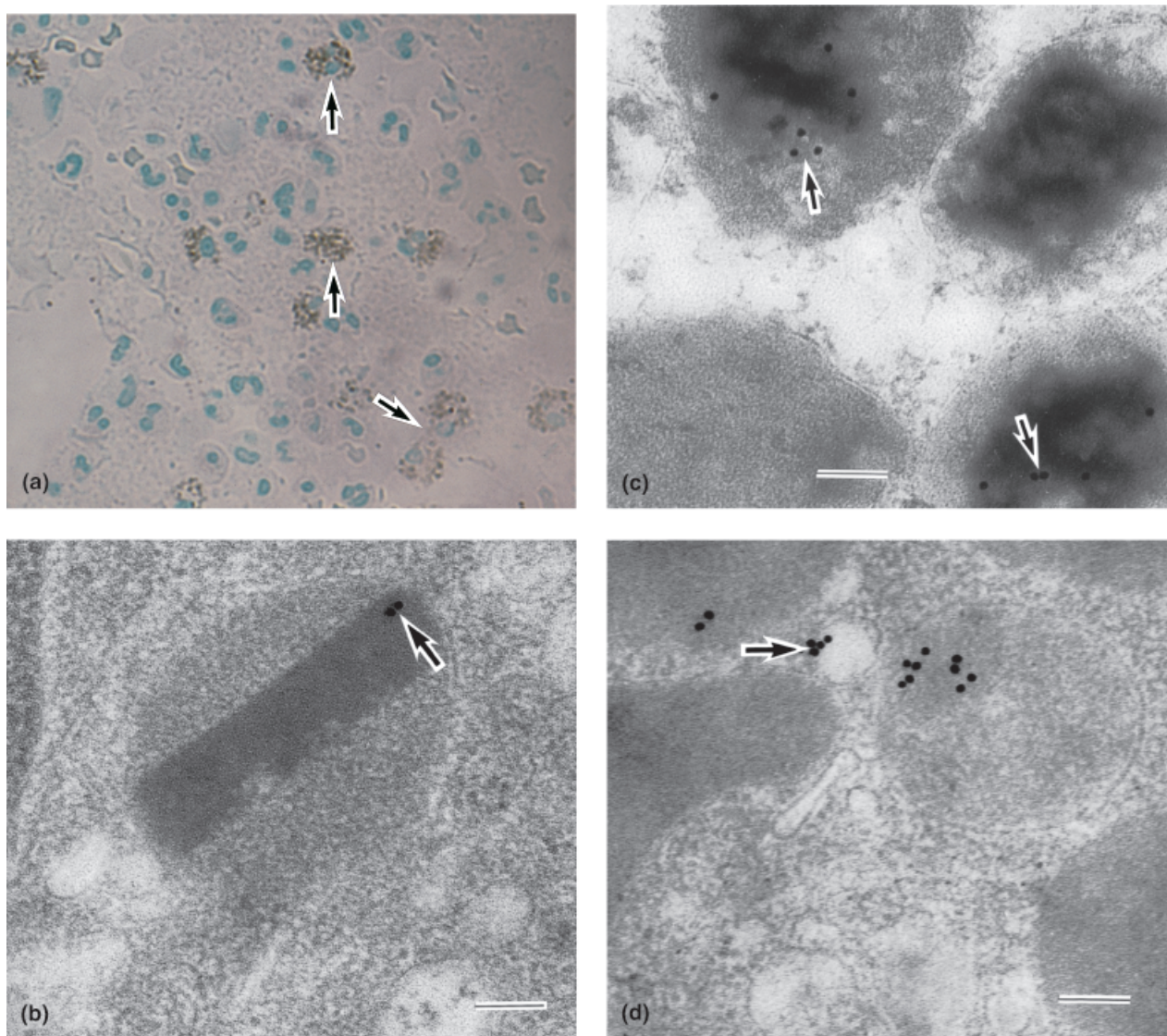


Fig. 1. NGF immunoreactivity in the human peripheral blood eosinophils. (a) Immunocytochemical staining of NGF in a smear of the peripheral blood. Eosinophils (arrows) are immunoreactive for NGF. The highest levels of immunoreactivity are localized in eosinophil granules. (Original magnification $\times 200$.) (b) An immunoelectron micrograph showing a few NGF-binding gold particles (arrow) that are present in the central core of a stable eosinophil granule. Bar: 0.1 μ m. (c) Homogeneous eosinophil granules characterized by granulolysis possess NGF-binding gold particles (arrows). Bar: 0.1 μ m. (d) NGF-binding gold particles are also localized in the intergranular vesicular structure (arrow). Bars: 0.1 μ m.

weak immunoreactivity for NGF. Various morphologies of specific granules within eosinophils were observed at the ultrastructural level, as previously reported [15]. Stable granules are composed of highly electron-dense central cores surrounded by a moderately electron-dense matrix with fine granular materials. Homogeneous granules, the activating and degranulating processes of eosinophils [16], were characterized by granulolysis where there are less distinct borders between the central core and the matrix. While eosinophils from control subjects mainly possessed stable granules, those from AD patients mainly contained homogeneous granules, and a small population of intragranular vesiculation, incomplete vacuolation and cytoplasmic vacuolization that results from eosinophil degranulation [15]. Post-embedding immunogold electron microscopy revealed that a small number of NGF-binding gold particles were localized in the central core of stable granules in eosinophils (Fig. 1b). In homogeneous granules, a relatively increased number of NGF-binding gold particles were observed (Fig. 1c). The number of NGF-binding gold particles varied considerably among specific granules as well as among eosinophils. NGF was also localized in the intergranular ductal or vesicular structures adjacent to specific granules in some eosinophils (Fig. 1d). The NGF antibody staining was specific since omission of the primary antibody, the use of an irrelevant monoclonal antibody of a similar isotype or absorption with excess NGF yielded no visible staining.

Number of eosinophils and NGF and MBP contents in eosinophil sonicates

There was a significant increase ($P < 0.01$) in the number of peripheral eosinophils in AD ($1285.2 \pm 203.6 \text{ mm}^{-3}$) patients vs. healthy controls ($370.2 \pm 124.5 \text{ mm}^{-3}$) (Fig. 2a). Freshly isolated eosinophils from 42 AD patients and from 31 control subjects were disrupted by sonication to determine levels of

NGF and MBP cell contents by ELISA and by RIA, respectively. Both NGF and MBP were detected in the sonicates of all subjects examined. The content of NGF in eosinophils of AD patients ($14.68 \pm 3.33 \text{ pg/mL per } 10^6 \text{ cells}$; mean $\pm$ SD) was significantly ($P < 0.005$) higher than those of controls ($3.15 \pm 1.38 \text{ pg/mL per } 10^6 \text{ cells}$) by the unpaired student's *t*-test (Fig. 2b). The MBP content in AD patients ($65.2 \pm 18.51 \text{ ng/mL per } 10^6 \text{ cells}$; mean $\pm$ SD) was significantly ($P < 0.05$) higher than those of controls ($40.60 \pm 7.39 \text{ pg/mL per } 10^6 \text{ cells}$) (Fig. 2c). There was a significant positive correlation between NGF and MBP contents in isolated eosinophils from AD patients ($Cc = 0.627$, $P < 0.005$; Fig. 3). In contrast, statistical analysis showed that there was no significant correlation between

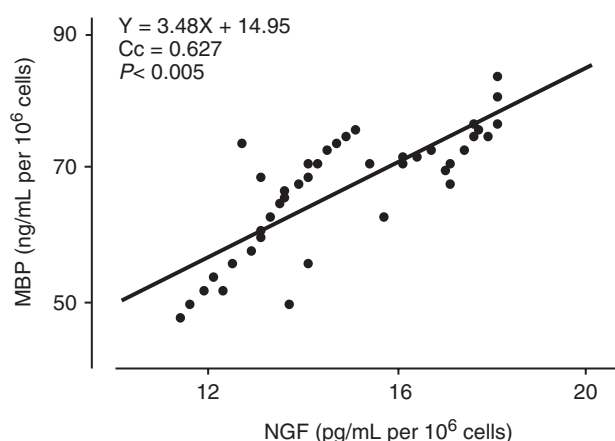


Fig. 3. Correlation between NGF and MBP contents in sonicates of freshly isolated eosinophils from the peripheral blood of patients with AD. The statistical correlation was examined by the Spearman rho test in a total of 42 patients with AD.

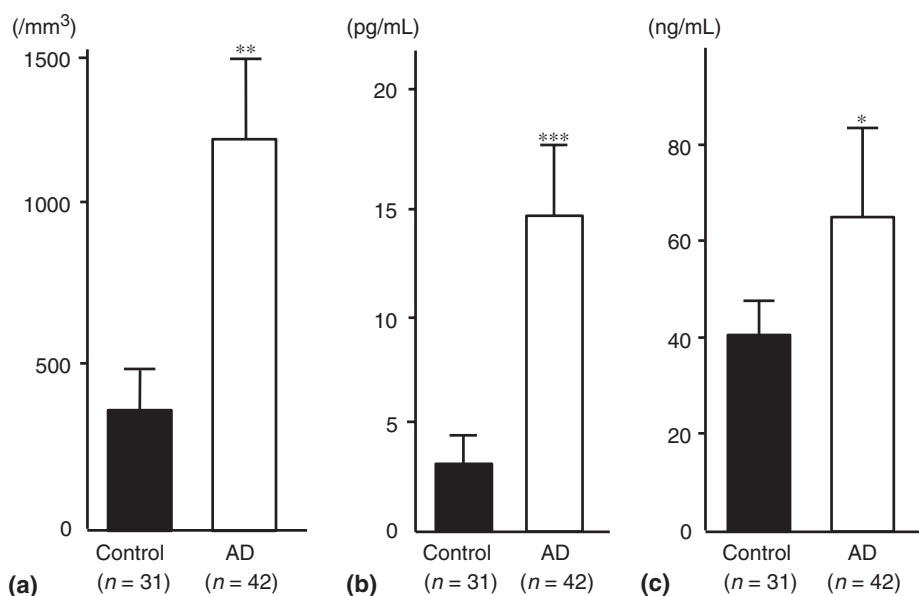


Fig. 2. Number of circulating eosinophils (a), and NGF (b) and MBP (c) contents in sonicates of freshly isolated eosinophils from the peripheral blood of patients with AD and of non-atopic healthy controls. Assays of NGF and MBP contents were performed in triplicate for each subject. Results reported are means $\pm$ SD. Statistical significance was determined using the unpaired student's *t*-test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ compared with the control.

NGF and MBP contents in eosinophil sonicates in the control group. No correlation was found between NGF content in eosinophils and the number of blood eosinophils in either groups.

DISCUSSION

This study demonstrates for the first time that: (1) NGF is localized in the central core of stable granules in human peripheral blood eosinophils, in homogeneous granules and in intergranular ductal or vesicular structures, (2) NGF content significantly increases in sonicates of freshly isolated eosinophils from AD patients compared with healthy controls, and (3) there is a significant positive correlation between levels of NGF and MBP contents in eosinophils of AD patients.

In AD lesional skin, mast cells, eosinophils and lymphocytes are active participants in promoting and perpetuating the inflammation through the complex action of cell-derived products and cytokines. Increases in serum levels of total and specific IgE, histamine, MBP, eosinophil cationic protein, as well as soluble factors [17], suggest not only the lesional but also the systemic involvement of various inflammatory cells in association with altered function of immune system of AD patients.

There is considerable evidence that NGF is able to exert a variety of effects on immune-inflammatory cells. It has been shown that NGF accumulates at the sites of inflammatory response and displays a potent chemoattractant activity for leucocytes [18]. Pro-inflammatory cytokines, such as TNF- α and IL-1, may vigorously stimulate NGF production, leading to the maintained increase of NGF during inflammation [19], which might result in an enhanced recruitment of inflammatory cells to the affected tissues. NGF modulates the synthesis and expression of neuropeptides which exert a wide control on the microenvironment, either enhancing or inhibiting different cellular functions [20]. NGF plays a role in cutaneous neurogenic inflammation by the augmentation of the secretion of neuropeptides, such as neurokinins, via a mast cell-dependent and a mast cell-independent pathway [21]. In addition to the observation that mast cells and Th2 lymphocytes are able to synthesize and release NGF [8, 9], it has been demonstrated by immunoenzymatic assay and reverse transcription PCR that human peripheral blood eosinophils store and produce NGF [10]. Attention and interest have recently focused on the role that NGF plays in allergic and immunologic diseases that include autoimmune rheumatic diseases, systemic sclerosis, asthma and vernal keratoconjunctivitis (reviewed in [22]). However, the pathogenic roles of eosinophil-derived NGF in those diseases have not been characterized. Although we have revealed that the plasma levels of NGF increase in AD and correlate significantly with its disease activity [11], the major source of circulating NGF in AD is still a matter of conjecture at present. The number of eosinophils in the peripheral blood and the NGF content in freshly isolated eosinophil sonicates were significantly increased in AD patients vs. controls, as shown in this study. A number of studies have shown that allergen-specific T cell clones of the Th2-type can be obtained from the peripheral blood, as well as from the skin lesions, of

AD patients, indicating the Th2 cell-predominance in AD. Mast cells are present constantly in the normal dermis and, in AD lesional skin, are increased in number as well as in size, activated and degranulated. *In vitro* studies have revealed that NGF stimulates T lymphocyte proliferation, induces mast cell proliferation, differentiation and degranulation [6], and activates eosinophils to elicit the release of eosinophil peroxidase [10]. Taken together, we postulate that the increased plasma concentrations of NGF in patients with AD [11] could partly result from the enhancement, activation and proliferation of eosinophils, Th2 cells and/or mast cells, all of which are able to synthesize, store and release NGF.

Our immunoelectron microscopic studies showed that NGF is localized in the central cores within normal eosinophil granules where MBP is also known to exist as a preformed mediator [23]. Upon eosinophil activation, specific granules of eosinophils undergo granulolysis (Fig. 1c), and basic proteins of eosinophils such as MBP are then released through the openings in the cell membrane connecting with intergranular ductal or vesicular structures (Fig. 1d) [16]. Besides the beneficial functions of the basic eosinophil granule proteins, such as their cytotoxic effects against parasites, eosinophil tissue infiltration and the extracellular deposition of eosinophil granules have been reported to lead to local tissue damage in various diseases [24]. MBP induces histamine release from rat mast cells and provokes cutaneous inflammatory reactions in human skin. Moreover, it has been reported that AD patients show significantly elevated serum levels of MBP compared with normal controls and that there is a significant correlation between serum MBP levels and the disease activity of AD [25]. In the present study, we showed that the MBP content within isolated eosinophils is highly increased in AD patients compared with healthy controls, indicating that specific granules of eosinophils in AD patients, most of which are in the activated stage, possess more increased amounts of MBP compared with those of normal subjects.

The finding that there is a statistically significant positive correlation between NGF and MBP contents in eosinophil sonicates in AD patients deserves particular interest. A recent report demonstrated that mast cells obtained from four different tissue sources (such as skin) have high protein levels of MBP in their granules as measured by flow cytometry and confocal laser scanning microscopy, which indicates that mast cells can produce abundant MBP [26], which had previously been thought to be eosinophil specific. Taking into account the co-existence of MBP and NGF in eosinophils and in mast cell-specific granules, it is tempting to speculate that the biosynthesis and/or the activities of these two inflammatory mediators might have common features or act interdependently in exacerbating inflammatory reactions in allergic diseases such as AD.

In conclusion, the findings reported here suggest that increased amounts of NGF are contained in eosinophils of the peripheral blood from AD patients compared with healthy controls, and that upon release with other chemical mediators such as basic proteins, could elicit inflammation and local tissue damage. This study may provide further evidence for the important role of eosinophils in the pathogenesis of AD, and a new insight into the neuro-inflammatory interactions that are likely to occur during

cutaneous allergic reactions in which eosinophils are involved. We believe that investigation of the functional role(s) of eosinophil-derived NGF may provide more insights into further understanding the pathogenesis of many allergic inflammatory disorders.

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